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DNA Ligase Mutants of *Escherichia coli*

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Abstract. A procedure is described for the isolation of *Escherichia coli* mutants with either excess or deficient DNA ligase activity. A mutant that overproduces DNA ligase supports the growth of ligase-defective (gene 30 mutant) T4 phages. Even T4 *r*II-gene 30 double mutants, which are able to grow in normal *E. coli*, cannot grow in cells deficient in DNA ligase. A functional DNA ligase, supplied either by the phage or the host, thus seems to be required for T4 growth. An *E. coli* strain that makes a temperature-sensitive DNA ligase becomes radiation-sensitive at high temperature, but otherwise grows normally and shows no obvious defect in DNA replication.

Covalent joining of DNA strands is thought to be an essential step in genetic recombination,[1] excision repair of irradiated DNA,[2] and DNA replication.[3] DNA ligase catalyzes the necessary reaction *in vitro*,[4–7] but has not been conclusively identified with any of these cellular functions.

Genetic analysis of DNA ligase has been restricted almost entirely to phage T4, where gene 30 was shown to be the structural gene of the T4-induced enzyme.[8] Gene 30 mutants in nonpermissive conditions do not multiply, show very restricted DNA replication, and fail to join pulse-labeled fragments of DNA into large molecules.[9–11] However, the interpretation of the phenotype of ligase-defective T4 is complicated by the activity of the host cell's DNA ligase. Thus, while DNA replication is blocked, formation of recombinant molecules[12] and repair of radiation damage[13] both persist. In addition, DNA replication and growth of T4 gene 30 mutants can be restored by secondary mutations in the *r*II gene of the phage[14–16]; it has not been clear whether DNA ligase activity is dispensable under these conditions.

For the further study of the functional role of DNA ligase, it seems profitable to characterize DNA ligase mutants in *Escherichia coli*. Such mutants have not been easy to find. A survey made some years ago of *E. coli* mutants defective in recombination, radiation repair, or DNA replication failed to uncover any strains with a defect in DNA ligase activity.[17] One *E. coli* mutant more recently reported[18,19] has temperature-dependent UV-sensitivity, temperature-sensitive growth and DNA replication, and a moderately reduced level of DNA ligase. Some experiments on the DNA ligase from this strain, *E. coli* TAU-bar ts-7, are included in our studies described below.

We have developed a screening procedure for the isolation of bacterial DNA ligase mutants. The method, based on the host cell's ability to support the

growth of ligase-defective T4 phages, provides mutants with either excess or defective DNA ligase activity. The properties of several mutants are discussed below.

Materials and Methods. Bacterial and bacteriophage strains: The parent strain of the bacterial mutants is *E. coli* N1071 *lac*$^-$ *galE*$^-$ *galK*$^-_{am}$*trp*$^-_{am}$*ara*$^-_{am}$ *T6*^{R} *su*$^-$ (λ*ind*$^-$), which is derived from strain MB-O of Russell *et al.*[20] From this strain we isolated, by methods described below, a mutation which leads to the overproduction of DNA ligase (*lop*8) and several ligase-defective mutations (*lig*2, *lig*3, and *lig*4). The resulting strains are: N1072 (*lop*8), N1252 (*lop*8 *lig*2), N1253 (*lop*8 *lig*3), and N1254 (*lop*8 *lig*4). These four mutants are also biotin-requiring, the requirement being an accidental result of the mutagenesis leading to N1072. *E. coli* TAU-bar ts-7[18] was obtained from C. Pauling.

T4 phage mutants *am*E605, *am*H39X, *ts*A80, and *ts*B20 (from the collection of R. S. Edgar) are amber and temperature-sensitive mutants in gene 30. T4*am*H39X*r*59 was obtained from H. Berger; *r*59 is an *r*II mutation. λ*ind*$^-$ is a noninducible mutant of phage λ.

Enzymes: *E. coli* exonuclease III and DNA polymerase[21] were gifts of I. R. Lehman and A. Kornberg, respectively.

Media: Unless specified, cultures were grown in tryptone broth (10 g of tryptone, 5 g of NaCl, 0.2 mg of thiamine hydrochloride per liter). Tryptone agar is the same medium solidified with 1.1% agar. M63 medium[22] was supplemented with thiamine·hydrochloride and biotin (each 0.1 μg/ml), Casamino acids (1 mg/ml), and L-tryptophan (20 μg/ml). T4 phages were assayed on EHA plates.[23]

Selection of DNA ligase mutants: *E. coli* mutants defective in DNA ligase were selected in two steps. First, mutants that permit the growth of ligase-defective T4 phages were isolated. At least two types of mutants may be expected: cells that replace the missing T4 function by overproducing DNA ligase, and cells that carry an amber-suppressor mutation, thereby permitting the synthesis of functional T4 DNA ligase. To exclude the second type, we used the method of Russell *et al.*[20] The parental strain, N1071, contains both a UDP-galactose epimerase mutation and a galactokinase amber mutation. It lacks a suppressor for amber mutations (*su*$^-$). If the cells are grown on a medium containing galactose, a mutation to *su*$^+$ permits the accumulation of UDP-galactose, which kills the cells. The strain was also lysogenized with phage λ, to exclude suppression by spontaneous *r*II mutants.

A culture of N1071 was mutagenized with nitrosoguanidine,[24] diluted, and spread on EMB-galactose agar[25] previously spread with 10^5 T4*am*E605 phages per plate. After a 24-hr incubation at 37°C, the plates were examined for red (partly lysed) colonies with irregular "nibbled" margins. Such colonies were purified and tested for their sensitivity to T4 ligase-defective phages. A small proportion (about one in fifty) proved to have the desired phenotype. One such mutant, *lop*8, was characterized in detail. (We use *lop* as an acronym for ligase overproducer.)

In the second selective step, we sought to isolate pseudorevertants that are overproducers of a defective DNA ligase. The *lop*8 strain was grown to a cell density of 10^8/ml in tryptone broth, and 4×10^7 cells were plated with 3×10^7 T4*am*H39X phages on tryptone–agar plates. The few colonies that appeared were purified and tested for their ability to support the growth of T4$^+$ but not T4*am*H39X. Three such mutants, *lop*8 *lig*2, *lop*8 *lig*3, and *lop*8 *lig*4, are discussed below.

Enzyme isolation: Cultures were grown to late log phase at 37°C in a yeast extract–phosphate–glucose medium[26] supplemented with biotin (0.1 μg/ml). *E. coli* TAU-bar ts-7 was grown at 32°C in the same medium with 10 μg/ml thymine added. Packed cells were distrupted with glass beads, and DNA ligase was purified (to fraction VI) by the procedure of Zimmerman and Oshinsky.[27] A batch of enzyme from *lop*8 *lig*4 was also carried through the phosphocellulose step (fraction VII).

DNA ligase assays: Two assays were used. (1) The conversion of poly dAT to a circular form was measured by the method of Modrich and Lehman,[28] with the addition

of *E. coli* tRNA (10 μg/ml) to minimize activity of endonuclease I in crude fractions. In the assay of purified fractions, 0.02 M potassium phosphate, pH 8.0, was substituted for Tris buffer.[27] This substitution did not change the activity of cruder fractions, but increased the apparent activity of fractions VI and VII, especially of mutant enzymes, several-fold. One unit of activity is defined as formation of 100 nmol of acid-precipitable dAT per 30 min.[28]

(2) The adenylylation of DNA ligase by radioactively labeled NAD was measured as previously described.[27] The assay was adapted for use with crude cell extracts by first passing the extracts over a Sephadex G-50 column (in 0.05 M Tris·HCl (pH 8.0), 20 μM EDTA) to remove compounds of low molecular weight, and by including 1 μM nicotinamide mononucleotide in the assay. Even in crude extracts of *E. coli* this assay appears to be specific for DNA ligase.

Pulse-labeling experiments: For these experiments the bacterial strains were made thymine-requiring by transduction with phage P1 grown on *E. coli* CR34. Most experiments were performed at 42°C, to increase the difference in DNA ligase activity between wild type and mutant strains.

Cells were grown at 42°C in supplemented M63 medium with [^{14}C]thymine added (40 μM and 4 Ci/mol). At a cell density of 4×10^8/ml, the culture was chilled, centrifuged, and resuspended in an equal volume of the same medium without thymine. A 5-ml aliquot was stirred for 5 min at 42°C, then [^{3}H]thymidine (0.4 μM and 17 Ci/mmol) was added. After 15 sec either the incubation was stopped or a 2000-fold excess of unlabeled thymidine was added, followed by 1–5 min further incubation. The incubation was stopped by dumping the culture into an ice slurry containing 0.01 M NaCN. After centrifugation, cells were lysed by resuspending the pellet in 0.1 M NaOH–0.01 M EDTA and incubating for 30 min at 37°C. An aliquot was layered onto a 25-ml, 5–20% alkaline sucrose gradient[4] (above a 3-ml shelf of 80% alkaline sucrose) and centrifuged for 10 hr at 22,500 rpm in a Spinco SW-25 rotor. Fractions were collected and acid-precipitated, and their radioactivity was determined in a Nuclear-Chicago Mark I scintillation counter.

Results. A mutant that overproduces DNA ligase: Among several mutants of N1071 selected for supporting the growth of ligase-defective T4 phages, the *lop*8 mutant was clearly the most "permissive." Only this mutant is discussed here.

Both amber and temperature-sensitive T4 gene 30 mutants plate on the *lop*8 mutant with high efficiency, while they plate on the parental strain very poorly (Table 1). The effect of the mutation is also apparent in the phage yield per infected cell. The yield of T4*am*H39X is 50-fold higher in the *lop*8 mutant than in N1071. The suppression is specific for gene 30 mutants; amber mutants

TABLE 1. *Growth of T4 gene 30 mutants on DNA ligase mutant strains of E. coli.*

	Efficiency of plating of:			
E. coli strain	T4*am*E605 or *am*H39X	T4*ts*A80 or *ts*B20 (at 42°C)	T4*am*H39X*r*59	Yield of T4*am*H39X per infected cell
N1071	2×10^{-4}	1×10^{-3}	1.0	5
*lop*8	0.9	1.0	0.8	250
*lop*8 *lig*2	$<10^{-5}$	...	$<10^{-4}$	0.3
*lop*8 *lig*3	7×10^{-5}	...	0.01	0.8
*lop*8 *lig*4, 32°C	0.8	...	0.9	90
*lop*8 *lig*4, 42°C	3×10^{-5}	...	1×10^{-3}	1

Efficiencies of plating were determined with log-phase cells, on EHA plates, and at 37°C unless specified. The efficiency of plating of amber mutants on the permissive strain *E. coli* CR63, and of temperature-sensitive mutants on *E. coli* B at 25°C, was taken as unity. Strains cured of phage λ were used for plating T4*am*H39X*r*59. The phage yield per infected cell was determined by infecting cultures with a low multiplicity of T4*am*H39X, removing unadsorbed phage with antiserum, and assaying infective centers before and after lysis of the cells.

in several other genes, including some involved in DNA replication, plate with an efficiency less than 10^{-4} on either N1071 or *lop*8. Mutants thus tested, with their gene assignments[29] in parentheses, include: *am*E315 (32), *am*N91 (37), *am*N122 (42), *am*B22 (43), *am*E4304 (43), and *am*N82 (44). The *lop*8 mutation is thus a gene-specific, allele-nonspecific, suppressor of T4 gene 30 mutants.

The suppression is effected by an increase in the host cell's DNA ligase activity. In crude extracts of the *lop*8 mutant there is 4–5 times as much DNA ligase activity as in the parental strain (Table 2). Since the same increase appears

TABLE 2. *DNA ligase activity in extracts of E. coli mutants.*

Strain	dAT-joining assay (units/mg)		Adenylylation assay (pmol/mg)	
	30°C	42°C	30°C	42°C
N1071	1.8	2.7	2.6	2.6
*lop*8	7.9	11.0	11.2	10.9
*lop*8 *lig*2	0.7	0.2	0.5	0.5
*lop*8 *lig*3	0.9	0.4	1.0	1.1
*lop*8 *lig*4	2.7	0.1	9.5	10.3

Extracts of cells grown at 37°C were assayed at the indicated temperature.

both in the dAT-joining assay (which measures the catalytic rate of the enzyme) and in the adenylylation assay (which in effect titrates the enzyme content of the extract), it seems that the catalytic activity per enzyme molecule is unchanged. We surmise that *lop*8 makes an increased amount of an unaltered DNA ligase. Consistent with this interpretation, the partly purified *lop*8 enzyme shows the same response to assay temperature as wild type enzyme (Fig. 1).

Mutants with a defective DNA ligase: Three pseudorevertants of the *lop*8 mutant were characterized. T4 gene 30 amber mutants plate on the *lop*8 *lig*2 and *lop*8 *lig*3 strains with even lower efficiency than on N1071 (Table 1), while T4$^+$ plates normally on all. There is a corresponding depression of the yield of T4*am*H39X per infected cell in *lop*8 *lig*2 and *lop*8 *lig*3.

In the *lop*8 *lig*4 strain, growth of T4 gene 30 mutants is temperature-sensitive. Below 33°C these phages grow almost as well as in *lop*8, while at 42°C phage growth is drastically impaired.

DNA ligase activity in these strains runs parallel to their ability to support the growth of ligase-defective T4 phage. In extracts of *lop*8 *lig*2 and *lop*8 *lig*3 strains, dAT-joining activity is depressed several-fold below that of N1071, and is somewhat temperature-sensitive (Table 2). The activity in a *lop*8 *lig*4 extract is strongly temperature-sensitive, being 30-fold lower at 42°C than at 30°C. That the depressed ligase activity in these mutant extracts is not due to an inhibitor was shown by mixing the extracts with purified DNA ligase. In no case was there more than 2-fold inhibition at the highest level of extract (100 μg/ml protein).

It is worth noting that the AMP-binding capacity of the mutant ligases is independent of temperature. In particular, the *lop*8 *lig*4 extract maintains at 42°C the elevated AMP-binding capacity characteristic of *lop*8, although the dAT-joining activity is greatly decreased (Table 2).

Further confirmation that DNA ligase is defective in these mutants was obtained from assay of partly purified fractions. Enzyme purified 50- to 80-fold from *lop*8 *lig*2 and *lop*8 *lig*4 maintains the same temperature sensitivity seen in crude extracts. The ratio of activity of the *lop*8 *lig*4 enzyme to wild type enzyme drops 100-fold between 20 and 46°C (Fig. 1). DNA ligase from the *lop*8 *lig*4 mutant, further purified by phosphocellulose chromatography (2000-fold overall purification), was equally temperature-sensitive.

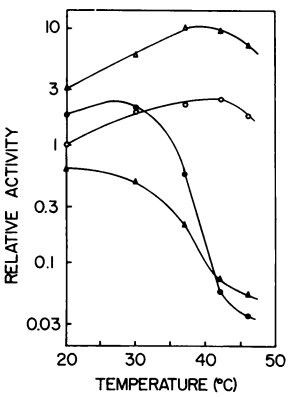

FIG. 1. Dependence of DNA ligase activity on temperature of assay. Source of enzyme: ○, N1071; △, *lop*8; ▲, *lop*8 *lig*2; ●, *lop*8 *lig*4. Extracts were purified 50- to 80-fold (to fraction VI) and assayed by the dAT-joining method. Specific activities are calculated relative to that of the N1071 enzyme at 20°C.

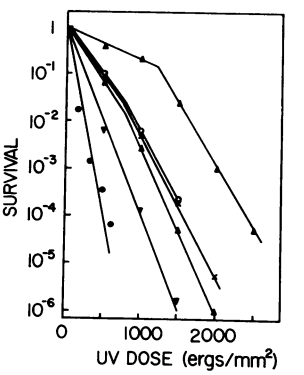

FIG. 2. UV survival curves of *E. coli* ligase mutant strains. Cultures were grown at 37°C to 4 × 10^8 per ml, centrifuged, diluted 50-fold in buffer, and irradiated under a 15-W GE germicidal lamp. Samples were diluted, plated on tryptone broth plates, and incubated in the dark for 24 hr, at 37°C unless specified. △, N1071; ×, *lop*8; ▽, *lop*8 *lig*2; ▲ *lop*8 *lig*3; ○, *lop*8 *lig*4 at 32°C; ●, *lop*8 *lig*4 at 42°C.

Physiology of the mutants: All the mutants (*lop*8 and *lop*8 *lig*2, 3, and 4) grow at the same rate as the parental strain at temperatures up to 42°C, in either tryptone broth or supplemented M63 medium. At 44°C, the *lop*8 *lig*4 strain still grows normally in supplemented M63; growth in tryptone broth is somewhat abnormal, with formation of "snakes" and with reduced viability.

There are some differences in UV-sensitivity among the mutants (Fig. 2). Even the *lop*8 mutant is somewhat more UV-sensitive than its parent. Since *lop*8 was isolated after drastic mutagenesis with nitrosoguanidine, which is known to produce multiple mutations, a correlation of the UV-sensitivity with overproduction of DNA ligase must await genetic analysis of this strain.

The UV-sensitivity of *lop*8 *lig*4 increases markedly with temperature. Up to 37°C this strain is no more sensitive than *lop*8, but at 42°C its survival after irradiation is much reduced. In this mutant it seems plausible to associate the temperature-dependent UV-sensitivity with the temperature-sensitive DNA ligase; since *lop*8 *lig*4 is a spontaneous mutant of *lop*8, the probability of two changes in closely related properties resulting from independent mutations is quite small.

A number of coliphages have been tested for growth on the DNA ligase mutants. Phages P1, P2, 186, λ*imm*^{21}, T4, and T7 plate as efficiently on the mutants as on the parental strain. (T7*am*13, a ligase amber mutant isolated and kindly supplied by Y. Masamune and C. C. Richardson, does not plate on the ligase-defective *E. coli* strains, although it plates on normal *E. coli*.)

Growth of a T4*r*II-gene 30 double mutant. Several workers have reported suppression of ligase-defective T4 mutants by secondary mutations in the *r*II locus.[14–16] It has been an open question whether the rII-gene 30 double mutants are able to dispense entirely with DNA ligase, or whether the *r*II mutation merely confers on a T4 ligase amber mutant the ability to grow with the low amount of ligase supplied by the host. As the last column of Table 1 shows, the second explanation is correct; a T4*r*II-gene 30 double mutant plates on the parental and ligase-overproducer strains, but not on the ligase-defective *lop*8 *lig*2 and *lop*8 *lig*3 mutants. In *lop*8 *lig*4, the plating of this phage is temperature-sensitive, as is the ligase activity. (For this experiment, the bacterial strains were cured of their λ prophage by superinfection with λ*imm*^{434}.)

Pulse-labeling experiments: To study DNA replication under conditions of reduced DNA ligase activity, we performed pulse-labeling experiments, of the type devised by Okazaki,[3] with the ligase-deficient mutants. As can be seen in Fig. 3, there is no gross difference in the behavior of the *lop*8 *lig*4 strain and

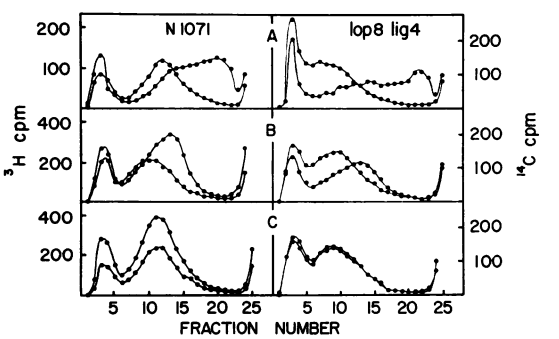

FIG. 3. Sedimentation of pulse-labeled DNA in an alkaline sucrose gradient. Cells prelabeled with [^{14}C]thymine were exposed to [^{3}H]thymidine for 15 sec at 42°C (panel *A*), then incubated with excess unlabeled thymidine for 1 min (*B*) or 5 min (*C*). ●, ^{14}C uniform label; O, ^{3}H pulse label. Sedimentation is from right to left.

N1071 at 42°C. In both strains, the DNA fragments first synthesized are quickly converted to a fast-sedimenting species. After a 1-min chase with unlabeled thymidine the pulse-labeled DNA sediments only slightly slower than the prelabeled DNA, and after a 5-min chase their sedimentation rates are indistinguishable. Similar results were obtained with *lop*8 *lig*2 at either 30 or 42°C.

We thus find normal joining of DNA fragments even with a greatly depressed DNA ligase activity.

The TAU-bar ts-7 mutant: We have measured DNA ligase activity in fractions from this strain that were somewhat more purified than those previously reported.[19] Enzyme purified to fraction IV[27] has 5–8% as much activity at 20°C as enzyme from wild type cells purified to the same stage. The activity of the ts-7 enzyme is only slightly temperature-sensitive, decreasing to 2–4% of wild type when assayed at 42°C. These assays, paralleling those in the orig-

inal report,[19] do not entirely account for the cessation of growth and of DNA joining in ts-7 at high temperature, since the *in vitro* DNA ligase activity of ts-7 at 42°C is no lower than that of *lop*8 *lig*4, but the latter strain is able to grow normally. It is possible that the intracellular level of DNA ligase in ts-7 at high temperature is further reduced by a temperature-sensitive rate of synthesis or by metabolic instability of the partly inactive enzyme.

Discussion. Our screening procedure for gene-specific suppressors of T4 gene 30 mutants yielded, by good fortune, an *E. coli* mutant with understandable phenotype. The *lop*8 strain replaces the missing T4 DNA ligase of gene 30 mutants by supplying an excess quantity of its own ligase. Since the suppression is very efficient, one must conclude that the DNA ligases of *E. coli* and T4 are functionally equivalent, and that the normal shift in cofactor requirement (from NAD for the *E. coli* enzyme to ATP for the T4 enzyme) has no deep physiological meaning. The 4- to 5-fold increase in ligase activity in *lop*8, which suffices to permit growth of gene 30 mutants, can be roughly correlated with the 2-fold increase[28] in total ligase activity after infection with normal T4. It is perhaps surprising that small changes in DNA ligase activity exert such a drastic influence on T4 multiplication.

Suppression of the T4 ligase defect by *r*II mutations in the phage may be explained in these terms as due to a change in the amount of DNA ligase activity required for growth, with T4*r*II needing perhaps a quarter as much DNA ligase as *r*II$^+$. Since T4 *r*II-gene 30 double mutants do not grow in a ligase-defective host, it is clear that they do require a certain minimum amount of DNA ligase for growth.

The increased DNA ligase level in *lop*8 could be the result of a mutation to increased enzyme synthesis (e.g. operator or promoter mutation), decreased degradation of the enzyme, or the generation of multiple gene copies. The third alternative can probably be excluded, because it would make subsequent mutation to ligase defectiveness unlikely. In the case of the *lop*8 *lig*4 mutant, comparison of the two types of ligase assay makes it clear that the cells contain the high ligase level of *lop*8, but that the enzyme is now defective. This strain is thus directly shown to be a double mutant.

The functional role of DNA ligase in *E. coli* is not yet clear. A considerable defect in DNA ligase activity can evidently be tolerated without obvious symptoms. Given a sufficient defect, as in *lop*8 *lig*4 or ts-7 at high temperature, an increase in UV sensitivity appears, but is not necessarily correlated with defective growth or DNA replication.

The conservative conclusion is that much more DNA ligase is made than is needed. It is still an open question, demanding further study, whether *E. coli* requires some minimal amount of DNA ligase for growth.

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